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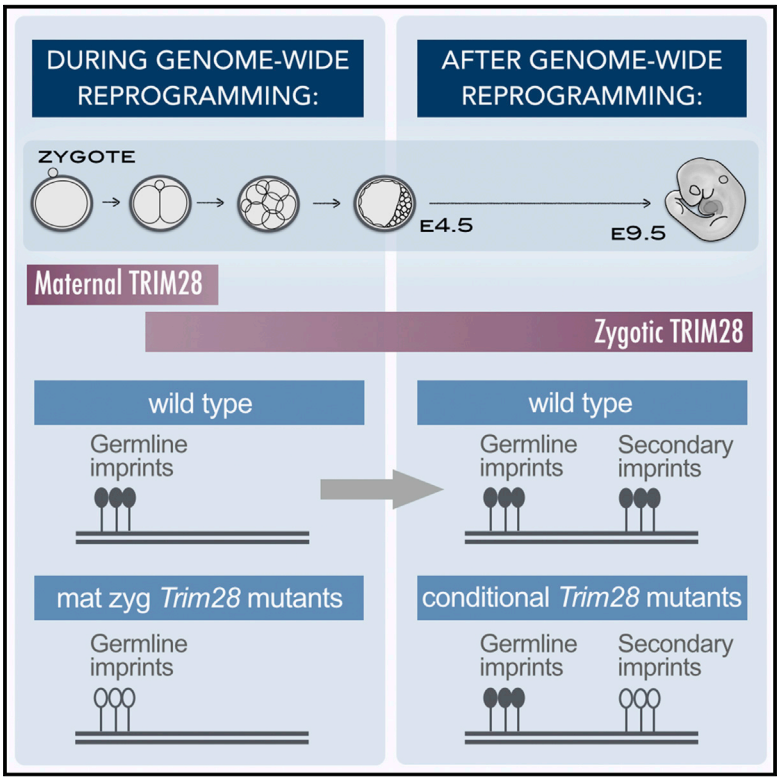
TRIM28 Controls Genomic Imprinting through Distinct Mechanisms during and after Early Genome-wide Reprogramming

Graphical Abstract



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In Brief

Alexander et al. demonstrate that the transcriptional repressor TRIM28 controls genomic imprinting through distinct mechanisms during and after early embryonic reprogramming. While TRIM28 is required for the maintenance of methylation at germline imprints during genome-wide demethylation, loss of TRIM28 at later embryonic stages causes hypomethylation at secondary, but not germline, imprints.

Highlights

- Zygotic TRIM28 is essential for imprinting control at many imprinted genes
- Loss of maternal and zygotic TRIM28 leads to fully penetrant loss of imprinting
- TRIM28 maintains germline imprints exclusively during genome-wide reprogramming
- Conditional *Trim28* mutants show hypomethylation at secondary DMRs

Alexander et al., 2015, Cell Reports *13*, 1194–1205
November 10, 2015
http://dx.doi.org/10.1016/j.celrep.2015.09.078

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TRIM28 Controls Genomic Imprinting through Distinct Mechanisms during and after Early Genome-wide Reprogramming

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http://dx.doi.org/10.1016/j.celrep.2015.09.078

SUMMARY

Genomic imprinting depends on the establishment and maintenance of DNA methylation at imprinting control regions. However, the mechanisms by which these heritable marks influence allele-specific expression are not fully understood. By analyzing maternal, zygotic, maternal-zygotic, and conditional *Trim28* mutants, we found that the transcription factor TRIM28 controls genomic imprinting through distinct mechanisms at different developmental stages. During early genome-wide reprogramming, both maternal and zygotic TRIM28 are required for the maintenance of methylation at germline imprints. However, in conditional *Trim28* mutants, *Gtl2*-imprinted gene expression was lost despite normal methylation levels at the germline *IG-DMR*. These results provide evidence that TRIM28 controls imprinting after early embryonic reprogramming through a mechanism other than the maintenance of germline imprints. Additionally, our finding that secondary imprints were hypomethylated in TRIM28 mutants uncovers a requirement of TRIM28 after genome-wide reprogramming for interpreting germline imprints and regulating DNA methylation at imprinted gene promoters.

INTRODUCTION

Genomic imprinting is a process that regulates the allele-specific expression of certain genes depending on their maternal or paternal inheritance. To date, about a hundred genes have been described to be imprinted, and defects in their expression have been associated with cancer and congenital disorders in humans (Lee and Bartolomei, 2013). Imprinted genes generally reside in gene clusters where their expression is controlled by regulatory sequences that are differentially methylated between the maternally and paternally inherited chromosomes. Two types of these differentially methylated regions (DMRs), germline and secondary DMRs, have been identified in mammals (reviewed in Ferguson-Smith, 2011). DNA methylation at germline DMRs is established during gametogenesis and is later maintained in the zygote after fertilization (Ferguson-Smith, 2011). In contrast, allele-specific methylation at secondary DMRs is established only after fertilization (Bartolomei et al., 1993; Bhogal et al., 2004; Brandeis et al., 1993; Ferguson-Smith et al., 1993; Szabó and Mann, 1995; Tremblay et al., 1995, 1997) and is dependent on imprinting marks at germline DMRs (Lin et al., 2003; Lopes et al., 2003; Srivastava et al., 2000, 2003).

Once established, methylation at germline and secondary DMRs is maintained in somatic cells throughout embryonic development (reviewed in Messerschmidt et al., 2014). First, germline DMRs resist the genome-wide DNA demethylation event that reprograms the oocyte- and sperm-derived genomes shortly after fertilization (Kafri et al., 1993). During this process, germline DMRs are protected from enzymatic removal of DNA methylation that characterizes the early stages of genome-wide reprogramming. Additionally, both during and after genome-wide reprogramming, DNA methylation at DMRs is maintained to prevent the replication-dependent dilution of methyl marks as the zygote starts to divide (Shen et al., 2014). Studies in mice have identified a few molecular mechanisms that prevent loss of DNA methylation at imprinted DMRs. The gene developmental pluripotency-associated 3 (*Dppa3*, also known as *stella* or *PGC7*) encodes a DNA binding protein that is maternally required to protect imprinted loci from enzymatic demethylation during genome-wide reprogramming (Bian and Yu, 2014; Gu et al., 2011; Nakamura et al., 2007, 2012). Additionally, DNA methyltransferase 1 (DNMT1) is required both maternally and zygotically to prevent replication-dependent loss of methylation at DMRs (Hirasawa et al., 2008).

While many studies support a central role for DNA methylation in imprinting control, the mechanisms by which these heritable chromatin marks are interpreted to regulate allele-specific expression are still not entirely understood. The prevailing view is that differential methylation between the maternal and paternal germline DMRs influences the allele-specific recruitment of factors that function in *cis* to influence transcription. However, while a few proteins are known to bind specifically to either methylated DNA or unmethylated DMRs, some of these proteins are not required for imprinting (Filion et al., 2006; Hendrich et al., 2001;

Monnier et al., 2013), and others only control imprinting at specific imprinted clusters (Balmer et al., 2002; Bell and Felsenfeld, 2000; Carr et al., 2007; Hark et al., 2000; Holmgren et al., 2001; Horike et al., 2005; Kanduri et al., 2000; Samaco et al., 2005; Szabó et al., 2000, 2004). Therefore, the current data argue that, rather than a universal mechanism to recognize methylated DNA, these epigenetic marks are interpreted in a locus-specific fashion to control transcription of nearby genes.

The transcriptional repressor TRIM28, also known as KAP1 and TIF1β, is required for genomic imprinting (Lorthongpanich et al., 2013; Messerschmidt et al., 2012; Quenneville et al., 2011). TRIM28 has been described to bind to the methylated allele of all known imprinting control regions, a recruitment that is dependent on the KRüppel Associated Box (KRAB) domain zinc finger protein ZFP57 (Li et al., 2008; Quenneville et al., 2011). However, the molecular mechanisms by which TRIM28 controls imprinting are still unclear. TRIM28 interacts with a variety of effector proteins, including Heterochromatin protein 1 (HP1; Lechner et al., 2000; Nielsen et al., 1999; Ryan et al., 1999; Sripathy et al., 2006), histone deacetylases (Schultz et al., 2001), histone methyltransferases (Frietze et al., 2010; Ivanov et al., 2007; Schultz et al., 2002), and the DNA methyltransferases, DNMT1, DNMT3a, and DNMT3b (Quenneville et al., 2011; Zuo et al., 2012). Whether one or more of these effectors is recruited by TRIM28 to control imprinting is not known. However, loss-of-function conditions for *Zfp57* or *Trim28* cause loss of DNA methylation and altered histone modifications at germline DMRs (Li et al., 2008; Lorthongpanich et al., 2013; Messerschmidt et al., 2012; Quenneville et al., 2011), indicating that these factors can, directly or indirectly, maintain epigenetic marks that preserve the imprinted status. Based on the facts that maternal depletion of TRIM28 causes loss of germline DMR methylation (Lorthongpanich et al., 2013; Messerschmidt et al., 2012) and that the zygote relies on maternally deposited proteins during the early stages of genome-wide reprogramming, it has been proposed that TRIM28 functions by protecting imprinted loci from DNA demethylation during this early reprogramming event (Messerschmidt et al., 2012). However, despite the fact that TRIM28 binds to all known germline imprints, depletion of maternal TRIM28 is only known to disrupt imprinting with variable penetrance at some imprinted clusters (Lorthongpanich et al., 2013; Messerschmidt et al., 2012). Variable effects on imprinting were also observed in *Zfp57* mutants, but the simultaneous loss of maternal and zygotic *Zfp57* caused more drastic effects than either mutant condition alone (Li et al., 2008), suggesting that effective maintenance of germline imprints requires both maternal and zygotic ZFP57.

To address the requirements of maternal and zygotic TRIM28 for genomic imprinting at different embryonic stages, we analyzed imprinted gene expression and DMR methylation in maternal, zygotic, maternal-zygotic, and conditional *Trim28* mutants. Results from these studies showed that zygotic *Trim28* is required to control imprinting at many imprinted loci, including imprinted clusters that were not previously identified in embryos depleted of maternal *Trim28*. Consistent with previous studies, our results support a role for maternal and zygotic TRIM28 in the maintenance of DNA methylation at germline DMRs during early embryonic reprogramming. Surprisingly, we also found that loss of TRIM28 at later embryonic stages disrupted allele-specific gene expression without affecting germline DMR methylation, providing evidence that TRIM28 controls imprinting through a molecular mechanism that is distinct from its role to preserve germline imprints during genome-wide reprogramming. Our analysis of conditional *Trim28* mutants also revealed hypomethylation at the *H19* and *Gtl2* promoters. Together, our results provide insight into the in vivo requirements of TRIM28 and the mechanisms that govern allele-specific expression of imprinted genes at different stages of embryonic development.

RESULTS

Zygotic TRIM28 Is Required for Proper Allelic Expression of Many Imprinted Genes

To determine whether zygotic TRIM28 is required for genomic imprinting, we evaluated imprinted gene expression in *Trim28*-null embryos ($Trim28^{L-}$; Cammas et al., 2000), and in homozygote mutants for $Trim28^{chatwo}$, a strong hypomorphic allele that causes developmental arrest at E8.5 and disrupts the protein stability and repressive activity of TRIM28 (Shibata et al., 2011). We first used qRT-PCR to test the levels of expression of imprinted genes in the *Igf2-H19* and *Dlk1-Gtl2* clusters (Figure 1A). This analysis revealed that the maternally expressed genes, *H19* and *Gtl2*, were upregulated in both $Trim28^{L-}$ and $Trim28^{chatwo}$ mutants, while the respective paternally expressed genes from these clusters, *Igf2* and *Dlk1*, were downregulated compared to wild-type littermate controls (Figures 1B and 1C).

To resolve whether abnormal *H19* and *Gtl2* expression levels in zygotic *Trim28* mutants were due to inappropriate biallelic expression, we sequenced cDNAs from embryos that contained SNPs distinguishing between the maternal and paternal alleles. While E8.5 wild-type embryos expressed imprinted genes monoallelically, $Trim28^{chatwo}$ embryos showed biallelic expression of *H19* and *Gtl2*, as well as *Airn*, *Rasgfr1*, *Gnas* (paternal isoform), *Snrpn*, *Peg10*, *Peg3*, and *Kcnq1ot1* (Figure 1D). These results demonstrate that expression of *Trim28* from the zygotic genome is required for allele-specific expression at many imprinted loci. Notably, our results show that loss of zygotic TRIM28 disrupts imprinted expression of *Gtl2*, which was previously only found to be marginally affected by loss of maternal TRIM28 (Messerschmidt et al., 2012). Thus, the analysis of $Trim28^{chatwo}$ mutants shows that TRIM28 has widespread requirements for controlling imprinting.

Loss of Imprinting Is Fully Penetrant in Maternal-Zygotic *Trim28* Mutants

Our analysis of allele-specific imprinted gene expression in single embryos revealed that loss of imprinting was partially penetrant in zygotic $Trim28^{chatwo}$ mutants (Figures 1D, 1E, and 2A–2C, column 4). This partial penetrance was not due to the hypomorphic nature of the *chatwo* mutation, since $Trim28^{L-}$ mutants also showed partially penetrant loss of imprinting (Figures 2A–2C, column 5). We hypothesized that maternal TRIM28, which is present during the early development of zygotic *Trim28* mutants, may account for the partial penetrance of imprinting defects in $Trim28^{L-}$ and $Trim28^{chatwo}$ embryos. To

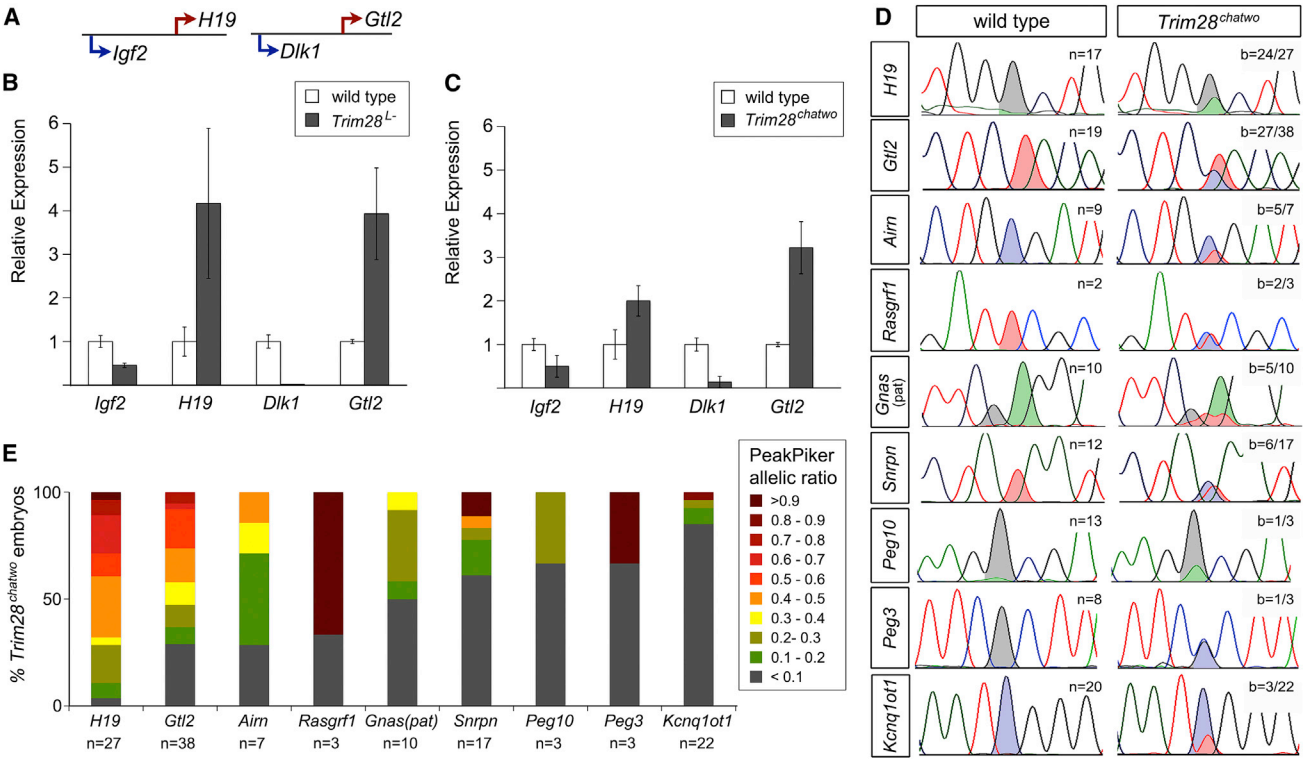

Figure 1. Imprinted Gene Expression in Zygotic *Trim28* Mutants

(A) Diagram of the *Igf2-H19* and *Dlk1-Gtl2* clusters, indicating maternally (red) and paternally (blue) expressed genes.

(B and C) Expression of imprinted genes in the *Igf2-H19* and *Dlk1-Gtl2* clusters as determined by qRT-PCR in pools of three to four E7.5 $Trim28^{L-}$ (B) and E8.5 $Trim28^{chatwo}$ (C) embryos. Data shown are normalized to β-actin and relative to wild-type controls. Error bars, SD from two biological replicates.

(D) Selected Sanger sequencing traces of cDNAs for *H19*, *Gtl2*, *Aim*, *Rasgrf1*, *Gnas* (paternal isoform), *Snrpn*, *Peg10*, *Peg3*, and *Kcnq1ot1* in individual E8.5 wild-type and $Trim28^{chatwo}$ embryos containing allele-specific SNPs (shaded peaks). All imprinted genes were analyzed in embryonic tissues, except for *Rasgrf1*, which is only imprinted in E8.5 extraembryonic tissues (Dockery et al., 2009). b = number of embryos with biallelic expression over the total number of embryos analyzed.

(E) Percentage of $Trim28^{chatwo}$ embryos with biallelic expression of imprinted genes as analyzed by Sanger sequencing and quantified using PeakPicker. Wild-type embryos showed PeakPicker allelic ratios between 0 and 0.1. Values higher than 0.1 were considered biallelic. A value of 1 corresponds to equal expression from both alleles. n = total number of embryos analyzed.

test this hypothesis, we generated embryos lacking both maternal and zygotic *Trim28* (*mzTrim28* mutants).

Maternal depletion of *Trim28* was accomplished with the use of a conditional allele of *Trim28* ($Trim28^{L2}$; Cammas et al., 2000) in combination with the *ZP3-Cre* transgene, which expresses *Cre*-recombinase from the oocyte-specific zona pellucida 3 (*ZP3*) promoter (de Vries et al., 2000). Mutants lacking both maternal and zygotic TRIM28 ($mzTrim28^{L-}$) arrested before implantation at the mid-blastocyst stage (Figure 2D). Some of these mutants formed a blastocele cavity similar to that of wild-type blastocysts but showed morphological abnormalities as compared with littermate controls, including slightly larger cells in the inner cell mass (n = 5/16, Figures 2F and 2F′). Other $mzTrim28^{L-}$ embryos failed to cavitate (n = 11/16) and occasionally displayed fragmented nuclei characteristic of cell death (n = 3/16, Figures 2G and 2G′). While the early lethality of these embryos prevented the analysis of imprinted gene expression in $mzTrim28^{L-}$ mutants, we found that embryos completely lacking maternal *Trim28* and carrying the hypomorphic $Trim28^{chatwo}$ allele zygotically ($mTrim28^{L-}$ - $zTrim28^{chatwo/L-}$ embryos), or embryos carrying the $Trim28^{chatwo}$ allele maternally and zygotically ($mTrim28^{chatwo/L-}$ - $zTrim28^{chatwo}$ embryos), survived past implantation and had a morphology and developmental arrest similar to that of zygotic $Trim28^{L-}$ mutants in dissections at E7.5. These two allelic combinations, from here onward referred to as hypomorphic *mzTrim28* mutants, were used to analyze the effects of loss of maternal and zygotic *Trim28* on imprinted gene expression.

In contrast to the partially penetrant biallelic expression of *Snrpn*, *H19*, and *Gtl2* observed in maternal or zygotic *Trim28* mutants (Figures 2A–2C, columns 2–5), we found that simultaneous depletion of maternal and zygotic *Trim28* in hypomorphic *mzTrim28* embryos disrupted imprinting in all embryos analyzed (Figures 2A–2C, columns 6 and 7; *Snrpn* n = 6; *H19* n = 9; *Gtl2* n = 8). One possible explanation for the complete penetrance of imprinting defects in hypomorphic *mzTrim28* mutants is that the amount of TRIM28 during the maternal to zygotic transition is critical for maintaining genomic imprinting during genome-wide reprogramming. In this scenario, loss of maternal TRIM28 could be partially compensated by zygotic TRIM28 and vice versa. However, it is also possible that the effects of lack of maternal and zygotic TRIM28 are additive and represent

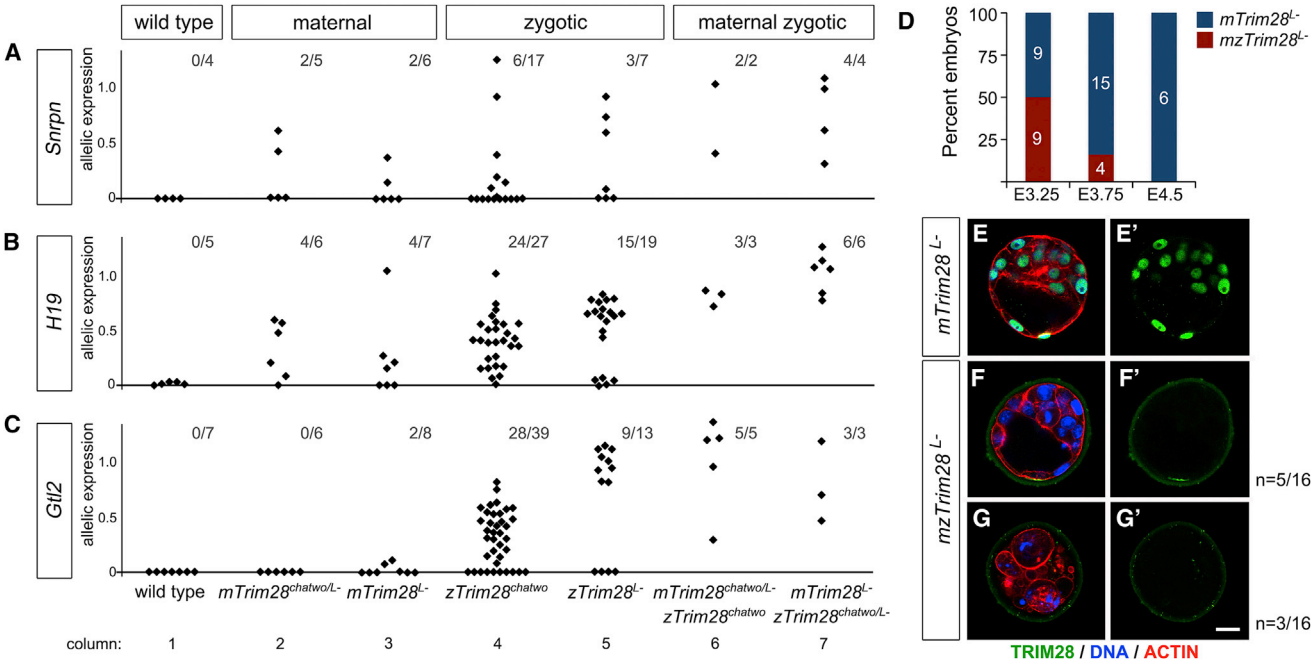

Figure 2. Analysis of Maternal, Zygotic, and Maternal-Zygotic *Trim28* Mutants

(A–C) Allelic expression in wild-type, maternal (*mTrim28*$^{chatwo/L-}$ and *mTrim28*$^{L-}$), zygotic (*zTrim28*^{chatwo} and *zTrim28*$^{L-}$), and hypomorphic maternal-zygotic (*mTrim28*$^{L-}$ - *zTrim28*$^{chatwo/L-}$ and *mTrim28*$^{chatwo/L-}$ - *zTrim28*^{chatwo}) *Trim28* mutants was analyzed at *Snrpn* (A), *H19* (B), and *Gtl2* (C) by Sanger sequencing and quantified with PeakPicker. Each diamond represents a single embryo. The fractional numbers indicate the number of mutants with biallelic expression over the total number of embryos analyzed. All embryos were analyzed at E8.5 except for *zTrim28*$^{L-}$ and hypomorphic maternal-zygotic mutants, which were analyzed at E7.5. Analysis of wild-type samples at E7.5 and E8.5 showed similar results (data not shown).

(D) Percentage of *mTrim28*$^{L-}$ (blue) and *mzTrim28*$^{L-}$ (red) mutants found in dissections at E3.25 (n = 18), E3.75 (n = 19), and E4.5 (n = 6).

(E–G′) Fluorescence staining of TRIM28 (green), DNA (DAPI, blue), and ACTIN (phalloidin, red) in *mTrim28*$^{L-}$ (E) and *mzTrim28*$^{L-}$ (F and G) blastocysts. TRIM28 localization (green channel) is shown separately in (E′) and (G′). Scale bar, 20 μm.

separate requirements for TRIM28 during early embryonic reprogramming and during later stages of embryogenesis. To resolve the specific roles of TRIM28 at different developmental stages, we analyzed the effects of *Trim28* depletion on DMR methylation in zygotic and conditional *Trim28* mutants.

DNA Methylation at Germline DMRs Is Disrupted in Zygotic *Trim28* Mutants

Because maternal TRIM28 has been previously implicated in the protection of germline DMRs from demethylation during early genome-wide reprogramming (Messerschmidt et al., 2012), we sought to evaluate whether zygotic TRIM28 is also required to maintain DNA methylation at germline DMRs. To this end, we used combined restriction-bisulfite analysis (COBRA), bisulfite sequencing, and quantitative pyrosequencing on *Trim28*$^{L-}$ and *Trim28*^{chatwo} embryos.

COBRA analysis showed loss of DNA methylation at the *H19*, *Snrpn*, and *Gtl2* germline DMRs in null *Trim28*$^{L-}$ mutants (Figures 3A–3C) suggesting that, similar to maternal TRIM28, zygotic TRIM28 is also required to preserve DNA methylation at germline DMRs. Consistent with the variable penetrance of imprinting defects in *Trim28*$^{L-}$ mutants (Figures 2A–2C), our COBRA experiments showed that hypomethylation at the *H19*, *Snrpn*, and *Gtl2* germline DMRs was also variable, with some *Trim28*$^{L-}$ embryos showing complete lack of methylation, while others only had partial or no effects (Figures 3A–3C). To further analyze the relationship between loss of germline DNA methylation and imprinted gene expression, we used pyrosequencing to quantify the allele expression ratio and germline DNA methylation level in an extensive collection of individual *Trim28*^{chatwo} mutants.

Consistent with the hypomorphic nature of the *Trim28*^{chatwo} allele and with the variability of allele expression ratios in these mutants (Figures 2A–2C), we found that *Trim28*^{chatwo} embryos showed partial loss of methylation at the *H19* and *Snrpn* germline DMRs (Figures 4A and 4B; Figures S1A, S1B, and S3). The extent of hypomethylation correlated with the ratio of *H19* biallelic expression in a collection of 26 individual *Trim28*^{chatwo} embryos (Figure 4A, $p < 0.05$), supporting that abnormal *Igf2-H19* imprinting in *Trim28*^{chatwo} mutants was caused by loss of DNA methylation at the *H19* germline DMR. Similarly, *Snrpn* allelic expression ratios also correlated with loss of methylation at the germline DMR (Figure 4B, $p = 0.001$). In contrast, we found that the levels of *Gtl2* germline DMR (*IG-DMR*) methylation in hypomorphic *Trim28*^{chatwo} mutants were not significantly different from those of wild-type littermates as analyzed with either COBRA (Figures S1C and S1D) or bisulfite sequencing (Figures 4C and 4D; Figure S3). Given that methylation of the *IG-DMR* was disrupted in null *Trim28*$^{L-}$ mutants (Figure 3C), the lack of effects in *Trim28*^{chatwo} embryos indicates that this hypomorphic allele provides enough TRIM28 function to allow

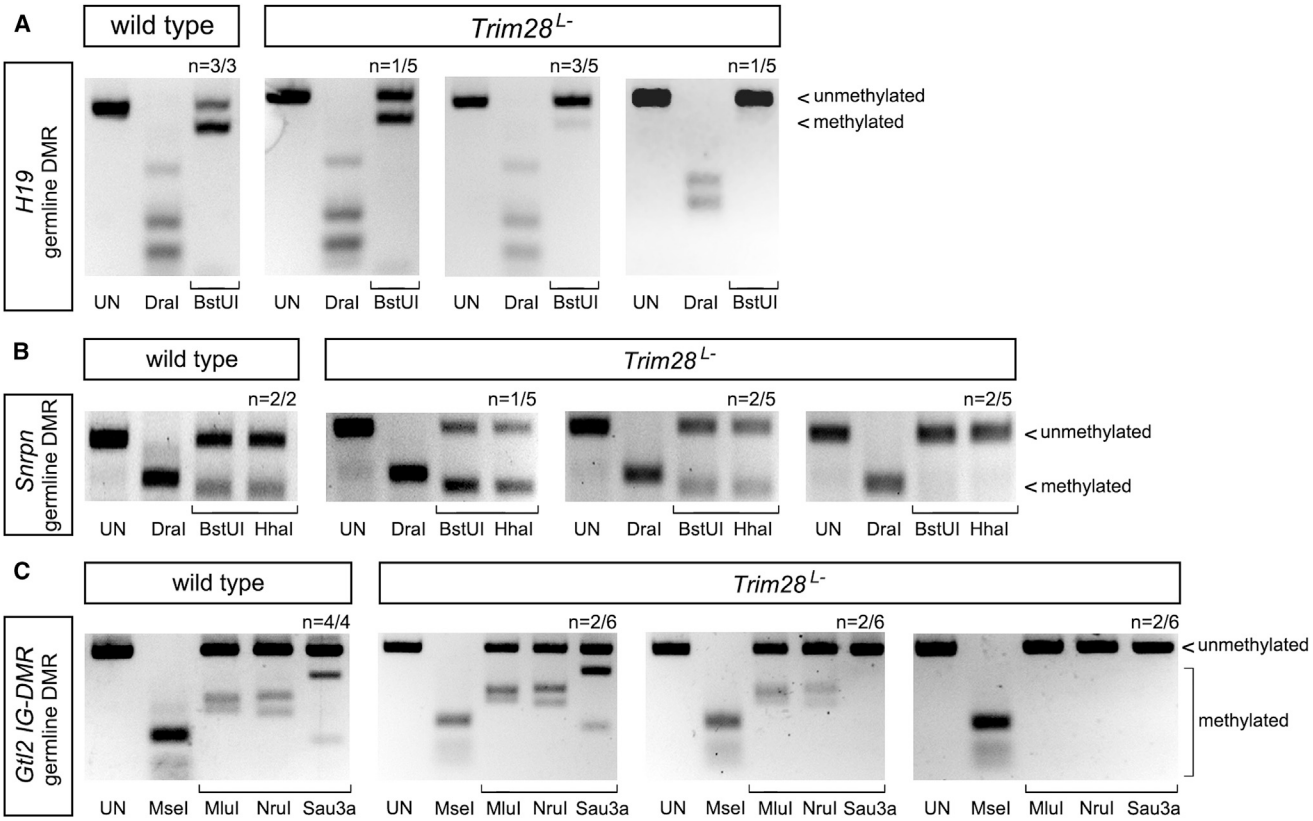

Figure 3. DNA Methylation at the *H19* and *Gtl2* Germline DMRs

DNA methylation at the *H19* (A), *Snrpn* (B), and *Gtl2* (C) germline DMRs was detected by combined restriction-bisulfite analysis (COBRA) in single E7.5 wild-type and *Trim28*$^{L-}$ embryos. n = number of embryos showing results similar to the one shown, relative to the total number of embryos analyzed. Restriction with DraI (A and B) and MseI (C) measured the efficiency of bisulfite conversion. All other restriction enzymes (lanes with brackets) only cut if the original sample was methylated. UN, undigested.

proper *IG-DMR* methylation maintenance. This result was nevertheless intriguing since the extent of *IG-DMR* methylation in *Trim28*^{chatwo} embryos did not correlate with the ratio of biallelic *Gtl2* expression observed in a collection of 16 *Trim28*^{chatwo} mutants (Figure 4C, $p = 0.56$). Therefore, these results provide evidence that, upon loss of TRIM28 function, germline DMR methylation is not sufficient to dictate imprinted expression of *Gtl2*. Consequently, our analysis of hypomorphic *Trim28*^{chatwo} embryos suggests that TRIM28 regulates imprinted expression at the *Dlk1-Gtl2* imprinted cluster through a molecular mechanism that is distinct from its function to preserve DNA methylation at germline DMRs.

TRIM28 Is Required for DNA Methylation at Secondary DMRs

Because allele-specific methylation at secondary DMRs has been proposed to maintain the imprinted status at some imprinted clusters (reviewed in John and Lefebvre, 2011), we hypothesized that biallelic expression of *Gtl2* in *Trim28*^{chatwo} embryos could be due to disrupted methylation of the secondary DMR located at the *Gtl2* promoter. To test this hypothesis, we used bisulfite sequencing and pyrosequencing to analyze DNA methylation at the *Gtl2* secondary DMR in hypomorphic *Trim28*^{chatwo} mutants. These experiments revealed that, despite normal levels of DNA methylation at the germline *IG-DMR* (Figures 4C, 4D, 5B, and 5C), *Trim28*^{chatwo} embryos had decreased levels of DNA methylation at the *Gtl2* secondary DMR as compared to wild-type littermates (Figures 5A–5C). While loss of DNA methylation at the *Gtl2* secondary DMR was not complete in *Trim28*^{chatwo} mutants, the extent of hypomethylation was highly correlated with the ratio of biallelic expression of *Gtl2* ($p < 0.001$, Figure 5D). Therefore, these results suggest that biallelic expression of *Gtl2* in *Trim28*^{chatwo} mutants could be due to loss of secondary DMR methylation. Additionally, the analysis of *Trim28*^{chatwo} embryos demonstrates a requirement of TRIM28 for DNA methylation at the *Gtl2* secondary DMR.

TRIM28 Has Separate Roles during and after Genome-wide Reprogramming

Since methylation at secondary DMRs is established after implantation (Brandeis et al., 1993; Ferguson-Smith et al., 1993; Sato et al., 2011), our finding that zygotic TRIM28 is required for DNA methylation at the *Gtl2* secondary DMR supports the conclusion that zygotic TRIM28 controls imprinting beyond the stages of early embryonic reprogramming. To

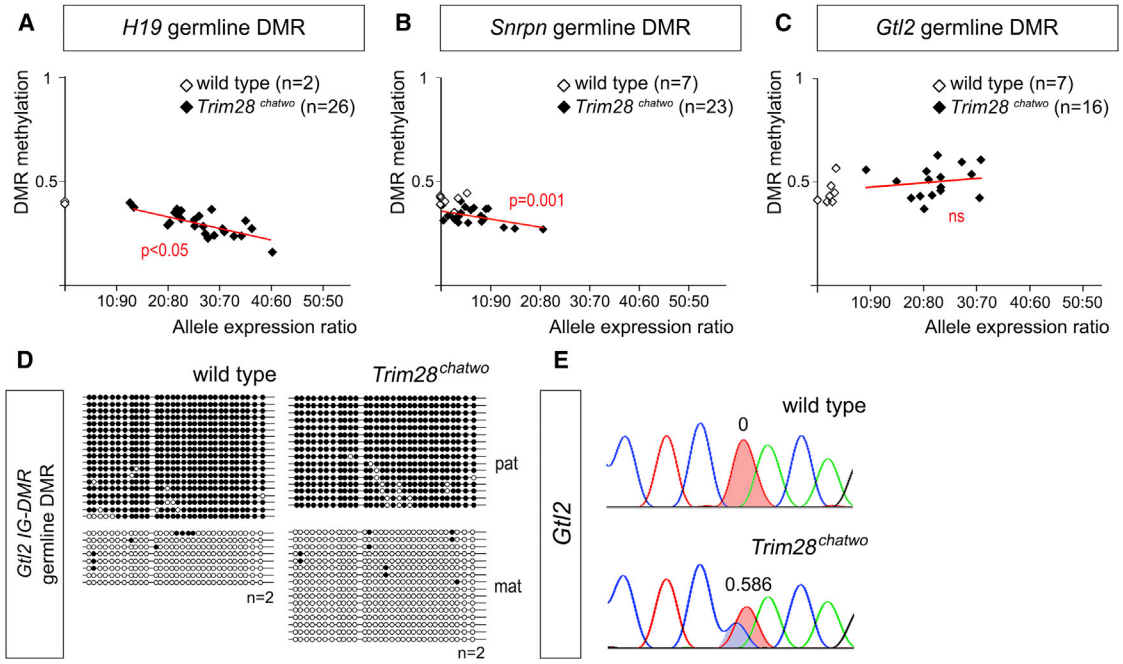

Figure 4. DNA Methylation at Germline DMRs

(A–D) DNA methylation at the *H19* (A), *Snrpn* (B), and *Gtl2* (C and D) germline DMRs was measured in single E8.5 wild-type and *Trim28*^{chatwo} embryos through pyrosequencing (A–C) and bisulfite sequencing (D). Plots in (A)–(C) represent the allelic expression ratio versus DMR methylation as measured by pyrosequencing. Figure S2 illustrates the relationship between allelic expression ratios quantified by PeakPicker and pyrosequencing. Red lines show the linear regression model for *Trim28*^{chatwo} embryos. p values (red) indicate the correlation between biallelic expression and DNA methylation. n = total embryos analyzed. (D) Representative bisulfite sequencing results for wild-type and *Trim28*^{chatwo} embryos; additional results are shown in Figure S3. Filled circles, methylated DNA. Empty circles, unmethylated DNA. Maternal (mat) and paternal (pat) chromosomes were identified by allele-specific SNPs.

(E) Sanger sequencing traces of cDNAs for *Gtl2* in the embryos analyzed in (D). Numbers indicate the PeakPicker allelic expression ratio.

further explore the roles of TRIM28 after this genome-wide reprogramming event, we tested the effects of *Trim28* loss of function using a conditional allele of *Trim28* (*Trim28*^{L2}) and the *Sox2Cre* transgenic line, which expresses *Cre* recombinase in all embryonic cells starting at implantation (Hayashi et al., 2002).

Analysis of imprinted gene expression using allele-specific SNPs revealed that *Gtl2* was biallelically expressed in *Sox2Cre*;*Trim28*$^{L-/L2}$ embryos (Figure 6A). Similar to our previous observations in *Trim28*^{chatwo} mutants, we found that *Sox2Cre*;*Trim28*$^{L-/L2}$ embryos had no significant differences in germline *IG-DMR* methylation (Figures 6B and 6C, $p = 0.75$) but showed a consistent decrease in DNA methylation levels at the *Gtl2* secondary DMR (Figures 6B and 6C, $p < 0.01$). Most significantly, biallelic *Glt2* expression in *Sox2Cre*;*Trim28*$^{L-/L2}$ embryos did not correlate with the small variations in DNA methylation at the *Gtl2* germline DMR (Figure 6D, $p = 0.32$; $n = 10$) but was highly correlated with loss of DNA methylation at the *Gtl2* secondary DMR (Figure 6E, $p < 0.001$; $n = 10$). Therefore, together with our previous analysis of *Trim28*^{chatwo} embryos, these results provide further support that the methylation at the *Gtl2* promoter is linked to the allele-specific silencing of *Gtl2.* Furthermore, since TRIM28 is only effectively depleted in *Sox2Cre*;*Trim28*$^{L-/L2}$ embryos after early embryonic genome-wide reprogramming (Shibata et al., 2011), our analysis of conditional *Trim28* mutants provides conclusive evidence that zygotic TRIM28 has separate roles to control imprinting during and after early embryonic reprogramming.

To determine whether TRIM28 also has separate roles during and after early embryonic reprogramming at other imprinted clusters, we analyzed DMR methylation and allele-specific expression of *H19* and *Snrpn* in *Sox2Cre*;*Trim28*$^{L-/L2}$ embryos. Similar to our findings at *Gtl2*, we found that there were no significant changes in the level of DNA methylation at the *H19* and *Snrpn* germline DMRs in *Sox2Cre*;*Trim28*$^{L-/L2}$ embryos (Figures 7A and 7B; Figure S4A). Consequently, our data argue against a general role of TRIM28 to prevent DMR methylation during the replication-dependent dilution of DNA methyl marks that takes place as cells undergo mitosis. Also similar to *Gtl2*, we found that *Sox2Cre*;*Trim28*$^{L-/L2}$ embryos showed significant hypomethylation at the *H19* secondary DMR (Figures 7A and 7B), demonstrating that the roles of TRIM28 to regulate the acquisition or maintenance of secondary DMR methylation are not exclusive to the *Gtl2* cluster. Interestingly, neither *H19* nor *Snrpn* was biallelically expressed in *Sox2Cre*;*Trim28*$^{L-/L2}$ mutants (Figure 7C; Figure S4B). Therefore, these results suggest either that the roles of TRIM28 after genome-wide reprogramming differ among imprinted clusters or that secondary DMR methylation has distinct roles for imprinting control at different imprinted loci. While more experiments will be required to uncover the mechanisms by which TRIM28 and secondary DMRs control imprinting after genome-wide

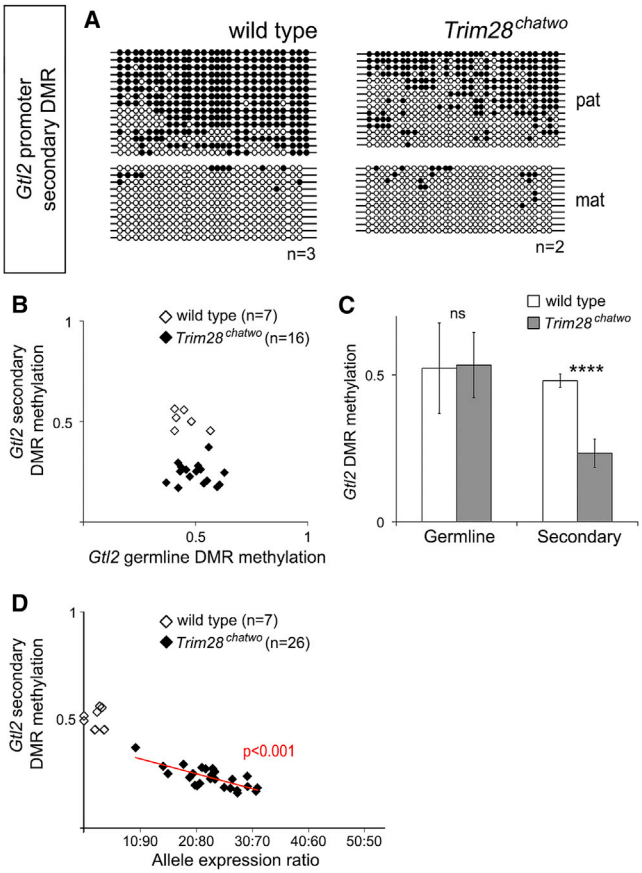

Figure 5. DNA Methylation at the *Gtl2* Secondary DMR Correlates with Biallelic Expression in *Trim28*^{chatwo} Mutants

DNA methylation at the *Gtl2* secondary DMR was measured in single E8.5 wild-type and *Trim28*^{chatwo} embryos by bisulfite sequencing (A) and pyrosequencing (B–D).

(A) Representative bisulfite sequencing results for wild-type and *Trim28*^{chatwo} embryos; additional results are shown in Figure S3. Bisulfite sequencing in (A) and Figure S3 shows results from *Trim28*^{chatwo} embryos that biallelically expressed *Gtl2*. Filled circles, methylated DNA. Empty circles, unmethylated DNA. Maternal (mat) and paternal (pat) chromosomes were identified by allele-specific SNPs.

(B) DNA methylation at the germline *IG-DMR* versus the *Gtl2* secondary DMR as measured by pyrosequencing in single wild-type and *Trim28*^{chatwo} embryos.

(C) Average DMR methylation for all wild-type and *Trim28*^{chatwo} embryos analyzed.

(D) *Gtl2* allele expression ratios versus *Gtl2* secondary DMR methylation as measured by pyrosequencing in single wild-type and *Trim28*^{chatwo} embryos. The red line in (D) shows the linear regression model for *Trim28*^{chatwo} embryos. The p value (red) indicates the correlation between biallelic expression and DNA methylation. The data represented in (B)–(D) include the same E8.5 wild-type and *Trim28*^{chatwo} embryos as shown in Figure 4C. n = number of embryos analyzed. Error bars, SD. Statistical significance was measured using a paired Student's t test: ns, not significant, ****$p < 0.0001$.

reprogramming, our experiments with *Trim28*^{chatwo} and conditional *Trim28* mutants provide conclusive evidence that zygotic TRIM28 is not required to maintain germline DMR methylation beyond the stages of early genome-wide reprogramming at both maternally and paternally imprinted loci.

DISCUSSION

Our study provides insights into how genomic imprinting is regulated during mammalian embryogenesis by uncovering distinct requirements for TRIM28 at different embryonic stages. First, we found that both maternal and zygotic TRIM28 are required to maintain DNA methylation at germline DMRs and that this function is exclusive to the first stages of embryonic development, when genome-wide reprogramming takes place. Furthermore, our experiments revealed that TRIM28 controls genomic imprinting at later stages of embryogenesis through a different mechanism that is independent of its role in maintaining DNA methylation at germline DMRs. The implications of these findings and the molecular mechanisms by which TRIM28 might regulate imprinting at these different stages of embryonic development are discussed below.

TRIM28 Has Widespread Requirements for Imprinting Control

Based on the finding that TRIM28 binds all known imprinting control regions (Quenneville et al., 2011), TRIM28 has been lauded as a master regulator of genomic imprinting. However, loss-of-function studies in embryos lacking maternal TRIM28 showed abnormal imprinted gene expression only in a subset of imprinted clusters (Messerschmidt et al., 2012). We found that imprinted gene expression in zygotic *Trim28* mutants was disrupted in all the maternally and paternally imprinted clusters we tested, including some loci that were not previously described to be disrupted by maternal *Trim28* depletion, such as *Airn*, *Rasgrf1*, *Gnas*, *Peg10*, *Peg3*, and *Kcnq1ot1*. Therefore, the results described in this study provide genetic evidence that *Trim28* has widespread requirements for genomic imprinting.

The Amount of TRIM28 Is Critical for Genomic Imprinting

The maternal-to-zygotic transition in mouse embryos takes place at the 2-cell stage (reviewed in Li et al., 2013), and zygotic expression of TRIM28 is detectable as early as the 4-cell stage (Messerschmidt et al., 2012), when embryos are still undergoing genome-wide demethylation (Smith et al., 2012). Therefore, it is tempting to speculate that maternal and zygotic TRIM28 function redundantly to protect imprinted loci from demethylation during this early genome-wide reprogramming event. Consistent with this hypothesis, we found that either maternal or zygotic *Trim28* mutants showed a partially penetrant loss of imprinting, but that simultaneous removal of both maternal and zygotic TRIM28 resulted in loss of imprinting in all the embryos analyzed.

The sensitivity of genomic imprinting to the amount of TRIM28 was also remarkable in zygotic *Trim28* mutants. Specifically, we show that complete removal of zygotic TRIM28 in null *Trim28*$^{L-}$ mutants caused loss of germline DMR methylation at *H19*, *Snrpn*, and *Gtl2*, but TRIM28 function in hypomorphic *Trim28*^{chatwo} mutants was sufficient to maintain normal levels of DNA methylation at the *Gtl2* germline *IG-DMR*. Because our previous observations support that the hypomorphic nature of the *chatwo* allele is largely due to a drastic decrease in TRIM28 protein levels (Shibata et al., 2011), we suspect that the different effects of the *Trim28*^{chatwo} allele on imprinting at

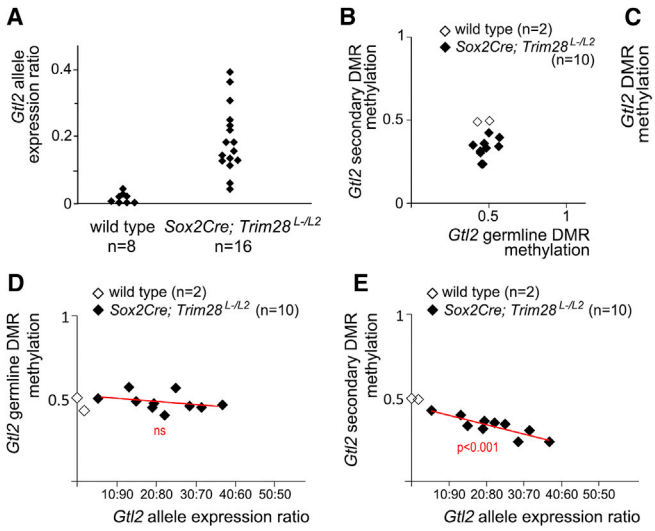

Figure 6. *Gtl2* DMR Methylation and Imprinted Gene Expression in *Sox2Cre*; *Trim28*$^{L-/L2}$ Embryos

(A) PeakPicker allelic expression ratios for *Gtl2* in single E8.5 wild-type and *Sox2Cre*;*Trim28*$^{L-/L2}$ embryonic tissues.

(B) Germline *IG-DMR* methylation versus *Gtl2* secondary DMR methylation in E8.5 wild-type and *Sox2Cre*;*Trim28*$^{L-/L2}$ embryonic tissues.

(C) Average DMR methylation in wild-type and *Sox2Cre*;*Trim28*$^{L-/L2}$ embryos. Error bars, SD. Statistical significance was measured using a paired Student's t test: ns, not significant, $^{**}p < 0.01$.

(D and E) *Gtl2* allele expression ratio versus *Gtl2* germline DMR methylation (D) and *Gtl2* secondary DMR methylation (E) in wild-type and *Sox2Cre*; *Trim28*$^{L-/L2}$ embryos. Red lines show the linear regression model for *Sox2Cre*;*Trim28*$^{L-/L2}$ embryos. p value indicates the correlation between biallelic expression and DNA methylation; ns, not significant. n = number of embryos analyzed.

the *H19*, *Snrpn*, and *Gtl2* might be a reflection of the specific dose-sensitive requirements for TRIM28 at these imprinted clusters. Together, these results indicate that genomic imprinting is particularly sensitive to the amount of TRIM28.

TRIM28 Maintains Germline DMR Methylation Exclusively during Genome-wide Reprogramming

The ability of TRIM28 to interact with the maintenance DNA methyltransferase DNMT1 in embryonic stem cells, and the fact that loss of *Zfp57* in embryonic stem cells (ESCs) leads to loss of germline DMR methylation (Quenneville et al., 2011; Zuo et al., 2012) have led to propose that TRIM28 maintains germline imprints throughout embryonic development (Messerschmidt, 2012). However, our finding that germline DNA methylation was not disrupted in conditional *Sox2Cre*; *Trim28*$^{L-/L2}$ mutants at either the *H19*, *Snrpn*, or *Gtl2* germline DMRs provides genetic evidence that TRIM28 is not required for replication-dependent maintenance of germline imprints after genome-wide reprogramming in vivo. Therefore, our results support the conclusion that TRIM28 maintains DNA methylation at germline imprints exclusively during the early stages of embryonic development.

DNA demethylation during genome-wide reprogramming is accomplished through both active and passive mechanisms (Shen et al., 2014). Active demethylation takes place through enzymatic oxidation of methylated cytosine residues by the ten-eleven translocation-3 (TET3) methylcytosine dioxigenase (Gu et al., 2011; Iqbal et al., 2011). Additionally, DNA methylation is passively lost through replication-dependent dilution of methylated cytosines, which is facilitated by the exclusion of the maintenance DNA methyltransferase DNMT1 from the nucleus during pre-implantation stages (Cardoso and Leonhardt, 1999; Doherty et al., 2002; Howell et al., 2001; Mertineit et al., 1998; Ratnam et al., 2002). Because TRIM28 has been shown to bind to the methylated allele of imprinting control regions (Quenneville et al., 2011), TRIM28 may interfere with active DNA demethylation by blocking the accessibility of TET3 to germline DMRs. However, it is also possible that TRIM28 interferes with passive mechanisms of DNA demethylation. In this respect, two studies have detected small amounts of DNMT1 in the nuclei of pre-implantation mouse embryos (Cirio et al., 2008; Kurihara et al., 2008). Given the ability of TRIM28 to interact with DNMT1 (Quenneville et al., 2011; Zuo et al., 2012), it is possible that TRIM28 might function by recruiting DNMT1 to germline DMRs during genome-wide reprogramming, ensuring that DNA methylation marks at these loci are perpetuated as the DNA replicates.

Separate Roles for TRIM28 during and after Early Embryonic Reprogramming

Perhaps the most striking result from our analysis of zygotic *Trim28* mutants was the finding that imprinting at the *Gtl2* cluster was disrupted in *Trim28*^{chatwo} embryos despite normal levels of methylation at the *IG-DMR*. This result provides evidence for a role of TRIM28 in the regulation of genomic imprinting that is independent of DNA methylation maintenance at germline DMRs. Because this role of TRIM28 is also supported by our analysis of conditional *Trim28* mutants, our data argue that the imprinting defects in *Trim28*^{chatwo} embryos are not due to a neomorphic effect of the *chatwo* allele but are rather caused by TRIM28 loss of function.

A Role for TRIM28 Interpreting Germline Imprints

While multiple studies support an essential role for germline DMRs in imprinting control (Lin et al., 2003; Thorvaldsen et al., 1998), there are still large gaps in our understanding of how differential methylation at these regulatory regions controls allele-specific expression of imprinted genes. One of the best characterized imprinting control regions is the *H19* intergenic germline DMR (Ferguson-Smith, 2011). This imprinting control region is recognized in a methylation-specific manner by the chromatin insulator CCCTC-binding factor (CTCF), which is known to influence chromatin topology and favor the interaction of the *H19* promoter with downstream enhancers (Bell and Felsenfeld, 2000; Engel et al., 2004; Hark et al., 2000; Murrell

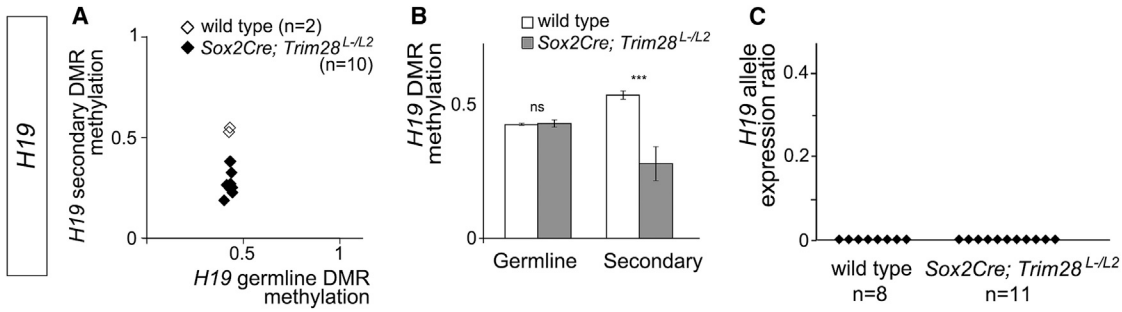

Figure 7. *H19* DMR Methylation and Imprinted Gene Expression in *Sox2Cre*;*Trim28*$^{L-/L2}$ Embryos

DNA methylation and allelic expression was measured at the *H19*-imprinted clusters by pyrosequencing (A and B) and Sanger sequencing (C).

(A) Germline DMR methylation versus secondary DMR methylation in E8.5 wild-type and *Sox2Cre*;*Trim28*$^{L-/L2}$ embryonic tissues.

(B) The same data are shown as the average DNA methylation levels at germline and secondary DMRs. Error bars, SD. Statistical significance was measured using a paired Student's t test: ns, not significant, ***$p < 0.001$.

(C) Allele expression ratios as quantified using PeakPicker.

et al., 2004; Szabó et al., 2000). The finding that CTCF also binds to other imprinted clusters (Fitzpatrick et al., 2007; Yoon et al., 2005) has brought some support toward an "insulator model" of imprinting regulation (Wan and Bartolomei, 2008). However, CTCF does not bind to all imprinting control regions (Carr et al., 2007). Consequently, the mechanisms by which germline DMRs function in *cis* to control allele-specific expression are likely specific of each imprinted locus. In this respect, the mechanisms that operate at the *Dlk1-Gtl2* germline *IG-DMR* to control imprinted gene expression are currently unknown (da Rocha et al., 2008). Chromatin immunoprecipitation experiments have failed to detect binding of CTCF, or methyl binding proteins MBD2 and MecP2, to the *Gtl2* germline *IG-DMR* (Carr et al., 2007). However, TRIM28 can bind the *Gtl2* germline *IG-DMR* in a methylation-specific fashion (Quenneville et al., 2011). By identifying TRIM28 as a factor that controls allele-specific *Gtl2* expression without disrupting germline DMR methylation, our studies provide genetic evidence supporting a role for TRIM28 in interpreting the epigenetic information inherited through the *Gtl2* germline *IG-DMR* to ultimately influence imprinted gene expression.

TRIM28 can recruit several histone-modifying enzymes (Schultz et al., 2001, 2002), and alterations in histone modifications are known to disrupt imprinted gene expression (Carr et al., 2007; Mager et al., 2003). TRIM28 is also known to mediate long-range transcriptional repression through heterochromatin spreading (Groner et al., 2010; Quenneville et al., 2012). Therefore, it is tempting to speculate that TRIM28 might regulate imprinting after early embryonic reprogramming by binding to the methylated *Gtl2* germline *IG-DMR* and spreading a repressive state through heterochromatin formation.

TRIM28 Is Required for Secondary DMR Methylation

DNA methylation at secondary DMRs has been proposed to control imprinting, but its role in regulating allele-specific expression is still controversial (reviewed in John and Lefebvre, 2011). Several studies support an instructive role of certain secondary DMRs for allele-specific expression. Secondary DMR methylation at the *H19* and *Gtl2* promoters has been shown to correlate with allele-specific silencing (Lin et al., 2003; Srivastava et al., 2000; Steshina et al., 2006; Thorvaldsen et al., 1998). Additionally, a study that conditionally deleted the paternal *Igf2-H19* germline DMR late in embryogenesis suggested that, once established, secondary imprints can maintain the imprinted status in the absence of the germline DMR (Srivastava et al., 2000). Since methylation at the *H19* and *Gtl2* promoters has been found to depend on the allele-specific methylation at germline DMRs (Lin et al., 2003; Srivastava et al., 2000; Thorvaldsen et al., 1998), a model has been put forward that secondary DMRs perpetuate the imprinted status inherited from germline DMRs. However, monoallelic expression of *H19* and *Gtl2* is established before DNA methylation is acquired at their secondary DMR promoters (Sasaki et al., 1995; Sato et al., 2011), arguing that secondary DMR methylation is a consequence of the imprinted status, rather than an instructive mechanism for allele-specific expression.

The fact that decreased levels of secondary DMR methylation in *Sox2Cre*;*Trim28*$^{L-/L2}$ embryos was highly correlated with biallelic expression of *Gtl2* provides additional data supporting the relationship between secondary DMR methylation and allele-specific expression. However, in *Sox2Cre*;*Trim28*$^{L-/L2}$ mutants, *H19* was not biallelically expressed despite significant loss of methylation at the *H19* promoter. These variable effects on different imprinted loci might be a reflection of the different mechanisms by which imprinting is regulated at specific clusters. For instance, it is possible that DNA methylation at the *H19* secondary DMR is dispensable for *H19* repression. Alternatively, it is possible that the roles of TRIM28 after genome-wide reprogramming differ among imprinted loci. Although our experiments cannot resolve whether methylation at secondary DMRs has an instructive role on imprinted gene expression or whether it is a secondary consequence of a previously established imprinted status, our results provide insight into the relationship between secondary DMR methylation and allele-specific expression of *Gtl2* and *H19*. The lymphoid-specific helicase LSH/HELLS is required for methylation of somatic imprints (Fan et al., 2005). However, LSH/HELLS seems to be required only at the *Cdkn1c*-imprinted locus (Fan et al., 2005). Therefore, by identifying a requirement for TRIM28 in the regulation of DNA methylation at *Gtl2* and *H19* somatic imprints, our results provide

an important contribution toward understanding the factors that control DNA methylation at secondary DMRs.

In conclusion, our analysis of maternal, zygotic, and conditional *Trim28* mutants not only provides additional insight about how TRIM28 maintains methylation at germline DMRs, but also uncovers a requirement of TRIM28 after genome-wide reprogramming for deciphering germline imprints and influencing secondary DMR methylation.

EXPERIMENTAL PROCEDURES

Mice

Trim28^{chatwo}, *Trim28*$^{L-}$, maternal *Trim28* deletion (*ZP3-Cre*;*Trim28*$^{L-/L2}$), and *Sox2Cre*;*Trim28*$^{L-/L2}$ mutants were obtained as previously described (Cammas et al., 2000; Messerschmidt et al., 2012; Shibata et al., 2011). *Trim28*^{chatwo} mutants were analyzed in a mixed CAST/Ei background, where mutants show developmental arrest at E8.5 (Shibata et al., 2011). To generate maternal *Trim28*$^{chatwo/L-}$ embryos, *Zp3-Cre*;*Trim28*$^{chatwo/L2}$ females were mated to wild-type males. For maternal-zygotic *Trim28* mutants, *Zp3-Cre*;*Trim28*$^{L-/L2}$ or *Zp3-Cre*;*Trim28*$^{chatwo/L2}$ females were mated to *Trim28*$^{L-/+}$ or *Trim28*$^{chatwo/+}$ males. For genetic backgrounds, SNPs and primers were used (see Table S1). Experiments involving mice were done according to standard operating procedures approved by Cornell's Institutional Animal Care and Use Committee.

Embryo Collection

Embryos were dissected in PBS containing 4% BSA. For post-implantation developmental stages (E6.5 and later), embryos were split into embryonic and extra-embryonic tissues that were processed separately for genotyping and imprinting analysis. In cases where allelic expression and DNA methylation were analyzed within the same embryo, the embryonic tissues were split and processed separately. At *Rasgrf1*, the embryonic tissues were used for genotyping, and extra-embryonic tissues were used for analysis of imprinted gene expression. For pre-implantation developmental stages, embryos were flushed from the uterus and used directly for immunofluorescence.

Gene Expression

Quantification of imprinted gene expression was tested by qRT-PCR on RNA samples extracted from independent pools of three to four E7.5 *Trim28*$^{L-}$ or E8.5 *Trim28*^{chatwo} embryos and wild-type littermates as previously described (Shibata et al., 2011). Allelic expression was detected by quantitative pyrosequencing and Sanger sequencing of RT-PCR products that amplified the SNP-containing region of the imprinted gene. Primer sequences and SNP positions are in Table S1. Allele expression ratios were quantified with PeakPicker (Ge et al., 2005).

Immunofluorescence

Preimplantation embryos were fixed in 4% paraformaldehyde and used for staining with TRIM28 antibody (Santa Cruz Biotechnology, sc-33186), phalloidin, and DAPI.

DNA Methylation

DNA was bisulfite converted using the EZ DNA methylation-direct kit (Zymo, D5020). For bisulfite sequencing analysis, PCR products of bisulfite-converted DNA were cloned using the TOPO TA cloning kit (Invitrogen, K450001), and individual clones were analyzed by Sanger sequencing. The efficiency of bisulfite conversion was >99%. Pyrosequencing was done as previously described (Wang et al., 2014). For primer sequences, see Table S1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.09.078.

ACKNOWLEDGMENTS

We thank Timothy Bestor, Mathieu Boulard, Paul Soloway, Patrick Murphy, and members of the laboratory for helpful discussions and comments on the manuscript; Joel Brown for help with graphical art; Florence Cammas, Shao-Cong Sun, and Jim Hagarman for mice and reagents; and Cornell's CARE staff for mice husbandry and care. This work was supported by NSF IOS-1020878 and NIH R01HD060581 to M.J.G.-G. and NIH S10RR025502 to the Cornell Institute of Biotechnology. K.A.A. was supported by NIH training grant 5T32HD057854-04 to Research and Career Training in Vertebrate Developmental Genomics at Cornell University.

Received: July 11, 2015
Revised: September 8, 2015
Accepted: September 25, 2015
Published: October 29, 2015

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